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Lack of Development of Huanglongbing in Seedlings from Citrus Seed

JAMES H. GRAHAM*[1], EVAN G. JOHNSON[1], DIANE B. BRIGHT[1], AND MICHAEL S. IREY[2]

[1]*University of Florida, IFAS, Department of Plant Pathology, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850*

[2]*United States Sugar Corp., Clewiston, FL 33440*

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Huanglongbing (HLB) was first discovered in Florida in 2005. In response, Florida citrus nurseries began treating rootstock seed trees located outdoors with insecticide applications to reduce risk of psyllid transmission of '*Candidatus* Liberibacter asiaticus' (*Las*), the putative causal agent. In 2008, a survey identified two 'Carrizo' citrange trees with symptoms of HLB. To assess the potential for seed transmission from HLB-affected seed source trees, assays of seedlings derived from seed extracted from symptomatic fruit were begun in 2006. From 2006 to 2008, 1557 seedlings germinated from 'Pineapple' sweet orange seeds from trees in Collier County were assayed by quantitative polymerase chain reaction (qPCR) using 16S rRNA gene primers. Of these seedlings, a single plant was positive for (*Las*+), although additional tests were negative. In 2009, no *Las*+ plants were detected among 332 'Murcott' tangor seedlings from trees in Hendry County. From nurseries in 2008, one *Las*+ seedling was detected in 290 seedlings from fruit located on symptomatic branches of two 'Carrizo' citrange trees, but its *Las*+ status was not confirmed after repeated testing. In 2009, a single *Las*+ result was obtained for one of 100 Cleopatra mandarin seedlings, whereas no *Las*+ seedlings were detected for 125 seedlings from seeds from two trees of 'Swingle' citrumelo, 649 seedlings from four trees of 'Kuharske' citrange, or 100 seedlings from one tree of 'Shekwasha' mandarin. Despite the occasional *Las*+ qPCR tests, no plants developed HLB symptoms. The most probable explanation for these results is transient transmission of *Las* from seed obtained from HLB-affected trees with no subsequent disease establishment.

Huanglongbing (HLB), greening, and yellow shoot are names for the most serious citrus disease in the world (Gottwald, 2010). HLB was first described in India early in the 20th century and later throughout Southeast Asia, but the disease was not known to be present in the Americas until 2004 and 2005 in Brazil and Florida, respectively. Symptoms of the disease include a distinctive mottle on fully expanded leaves (Gottwald et al., 2007). Infected shoots are stunted and branches gradually die back as the symptoms appear in other sectors of the tree. Fruit from affected branches may be lopsided and remain green at the stylar end of the fruit. Yield is reduced directly by fruit drop and indirectly by tree decline (Bassanezi et al., 2011). HLB reduces fruit size, weight, and other fruit quality variables, such as total soluble solids (TSS) content, acidity, and TSS/acidity ratio (Bassanezi et al., 2009). Affected trees produce fruit that can contain aborted and healthy-appearing seeds with coats that are infected with the pathogen (Tatineni et al., 2008). Symptoms of the disease are associated with the presence of bacteria in the phloem of infected trees as determined by polymerase chain reaction (PCR)-based assays (Jagoueix et al., 1994) and recently by metagenomic analysis (Tyler et al., 2009). In spite of numerous attempts, including recent ones (Davis et al., 2008; Sechler et al., 2009), the bacterium is not in culture and is unavailable to fulfill Koch's postulates. Analysis of the 16S ribosomal RNA gene of the HLB-associated bacteria has led to the recognition of three species of the '*Candidatus*' genus Liberibacter: '*Candidatus* Liberibacter asiaticus' (*Las*), '*Ca*. L. africanus', and '*Ca*. L. americanus' (Teixeira et al., 2005).

Transmission of *Las* through infected citrus seed has been reported (Tirtawidjaja, 1981) based on HLB-like symptoms in seedlings when apparently healthy seed were harvested from symptomatic fruit and planted. *Las* was also readily detected throughout HLB-affected fruit by qPCR (Li et al., 2009) and in the seed coat, but not the embryos of seed collected from HLB-affected fruit (Tatineni et al., 2008).

The planting of *Las*-free trees is a critical component of a comprehensive strategy to manage HLB (Gottwald, 2010). In Florida and Brazil citrus nurseries, pathogen-free trees are produced from propagations of budwood sources tested for the presence of *Las*. These trees are grown in houses that exclude citrus psyllids to assure that they are free of *Las* when planted. In contrast to the scion budwood, the rootstock trees used to produce seed for these trees are not protected from the citrus psyllids. A survey of Florida citrus nursery seedling blocks detected *Las*+ trees beginning in 2008 (Irey, unpublished data), identifying a risk that seeds used for propagation of rootstock seedlings could be infected with *Las*. Thus, if the pathogen is transmitted through seed, production of *Las*-free trees would be compromised. Also, citrus germplasm distributed as seed could act as a pathway for the potential introduction of *Las* into HLB-free regions.

The purpose of this work was to provide evidence for or against the risk of *Las* being transmitted through seed to seedlings.

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*Corresponding author; phone: (863) 956-8660; email: jhgraham@ufl.edu

Materials and Methods

Sampling of grove trees. In Nov. 2006, small, lopsided fruit were collected from 10 'Pineapple' sweet orange [*Citrus sinensis* (L.) Osb.] trees in severe decline (defoliation and branch dieback) from HLB in Collier County, Florida, and 415 seedlings were obtained from seed extracted from symptomatic fruit. In Jan. 2007, symptomatic fruit were collected from eight 'Pineapple' trees in the same location. Seeds from 'Pineapple' fruit were extracted and classified as healthy, off-colored gummy, or aborted. Healthy (359) and off-colored (344) seed and the resultant 703 seedlings were assayed. In 2008, 'Pineapple' seeds (439) were harvested from eight symptomatic trees in the same location in Collier County and healthy-appearing seeds (332) were obtained from small or misshapen fruit from five symptomatic 'Murcott' tangor (*C. reticulata* × hybrid) trees collected in Hendry County.

Sampling of seed source trees in citrus nurseries. In 2008, 290 seedlings were germinated from seeds from fruit located on symptomatic branches of two 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. × *C. sinensis*] seed trees from a commercial citrus nursery. In 2009 from one citrus nursery, 125 seedlings were germinated from seeds from two trees of 'Swingle' citrumelo (*P. trifoliata* × *C. paradisi* Macf.), 649 seedlings from four trees of 'Kuharske' citrange, 100 seedlings from one tree of 'Cleopatra' mandarin (*C. reticulata* Blanco). From a second citrus nursery, 100 seedlings were obtained from one tree of 'Shekwasha' mandarin (*C. reticulata*). For nursery samples in 2009, normal-appearing fruit were collected from *Las*+ branches as determined by PCR and gel electrophoresis (Jagoueix et al., 1996) from disease surveys carried out by the Florida Department of Agriculture and Consumer Services, Department of Plant Industry (DPI).

Processing of fruits and propagation of seedlings. Seeds were extracted from the fruit and seed coats removed and frozen for assessment later. Each seed with or without the seedcoat was germinated individually in 3.8 cm × 21 cm Cone-tainers (SC10 Super Cell; Stuewe & Sons, Corvallis, OR) containing Custom Citrus Fafard Mix (Contad Fafard, Inc., Agawam, MA). Plants were fertilized every 2 weeks with a liquid fertilizer containing an N–P–K ratio of 20:10:20 (Peters Professional; The Scotts Company, Marysville, OH) supplemented with minor elements. The seedlings were grown in a USDA Animal and Plant Health Inspection Service-approved secure growth room at 28 °C in 2006 and in 2007–2009, in a greenhouse with a temperature of 26 °C to 32 °C. Photosynthetically active radiation (*PAR*) was measured using a LI-185 Quantum Radiometer/Photometer (Lambda Instruments Inc., Lincoln, NE). The *PAR* value measured in the growth room was 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ over a 24-h photoperiod. In the greenhouse, the *PAR* was 300 to 570 $\mu E \cdot m^{-2} \cdot s^{-1}$ during natural photoperiods. Seedlings were observed regularly for blotchy leaf mottle, stunting, and chlorosis, typical symptoms of HLB.

PCR methods and identification of amplicons. Fully expanded leaves collected from seedlings at the 5- to 25-leaf stage (≈3 to 9 months after seedlings emerged from seed) and seedcoats were assayed with qPCR using primers based on Li et al. (2006) and the Southern Gardens Diagnostic Lab (SGDL) protocol for HLB testing as previously described (Irey et al., 2006). In the SGDL, a Ct value of less than 30 is considered positive and greater than 30 and less than 32 is considered questionable for the presence of Las. Seedlings that tested positive or were considered questionable by SGDL were subjected to further testing and the amplified DNAs were sequenced. The qPCR assay was repeated with both 16S rRNA and beta-operon primer sets and run for 50 cycles (Li et al., 2006; Wang et al., 2006). Ct values were converted to copies of target genes using a standard curve based on a serial dilution of plasmids containing target sequence. Nested PCR was performed with three sets of nested primer sets targeting the 16S rRNA (OI1/OI2c, CGO3f/CGO5r), beta-operon (MHO317/319, Bf2/Br2), and omp genes (OMP6f/OMP1r, OMP3f/OMP2r) (Ding et al., 2005; Hoy et al., 2001; Jagoueix et al., 1996; Zhou et al., 2007). Products from nested PCR were cloned using Qiagen PCR cloning kit. Plasmids containing the cloned PCR product were sent to the University of Florida, Interdisciplinary Center for Biotechnology Research (UF–ICBR) for sequencing.

Results

2006. From 415 seedlings sampled, Ct values for two plants were positive for *Las* and five plants were questionable. Of a subset of 59 seeds that produced seedlings, 14 had *Las*+ seed coats but no *Las*+ or questionable seedlings germinated from those seeds (Table 1). Retesting of leaf samples from the same seven plants yielded three plants with Ct values consistently at or below 32. After a third assay, leaves from a single plant continued to test positive by qPCR, but leaf tissue collected 6 months after the last positive finding tested negative.

Table 1. Number of seedlings grown from seeds of scion and rootstock trees affected by huanglongbing in Florida groves and citrus nurseries from 2006 to 2009 and status of those seedlings for detection of '*Candidatus* Liberibacter asiaticus' (*Las*) after PCR assay.

				No. of *Las*+ or questionable seedlings[z]			
					Assay		
Year	Genotype	No. of trees sampled	No. of seedlings germinated	Seedlings	After 2nd	After 3rd	After 4th
2006	'Pineapple' orange	10	415	7	3	1	0
2007	'Pineapple' orange	8	703	6	1	0	na[y]
2008	'Pineapple' orange	8	439	0	na	na	na
2008	'Murcott' tangor	5	332	0	na	na	na
2008	'Carrizo' citrange	2	290	1	0	na	na
2009	'Kuharske' citrange	4	649	0	na	na	na
2009	'Swingle' citrumelo	2	125	0	na	na	na
2009	'Cleopatra' mandarin	1	100	1	na	na	na
2009	'Shekwasha' mandarin	1	100	0	na	na	na

[z]Number of seedlings with Ct ≤ 32.
[y]na = not analyzed.

2007. Seeds extracted from 'Pineapple' fruit were classified as healthy (28%), off-colored gummy (29%), or aborted (43%). Healthy (359) and off-colored (344) seeds were planted and leaves from the resultant 703 seedlings were assayed by qPCR (Table 1). From this group of seedlings, six had Ct values less than or equal to 32. After a second assay of leaf tissue from the same plants, a single 'Pineapple' plant was a questionable *Las*+. After a third assay of leaves from the questionable *Las*+, the plant was negative.

2008. Four hundred thirty-nine 'Pineapple' and 332 'Murcott' tangor seedlings tested negative for *Las*. From 290 'Carrizo' citrange seedlings, a single *Las*+ was detected by nested PCR using 16S rRNA primers (Table 1). The amplicons from this assay were cloned, sequenced, and identified as *Las*. However, several follow-up tests of the same plant using the beta-operon qPCR and all nested primer sets listed above were negative.

2009. 'Swingle' citrumelo (125), 'Kuharske' citrange (649), 'Cleopatra' mandarin (100), and 'Shekwasha' mandarin (100) seedlings all tested negative, except for one questionable *Las*+ for a 'Cleopatra' seedling (Table 1). Subsequent detection from two DNA extractions from leaves of the same plant using the 16S primer/probe set produced Ct's ranging from 38.7 to 46.6, and the assay was negative using beta-operon primers (Wang et al., 2006) and multiple nested PCR primer sets. Two months later, midrib and bark extracts from the same plant tested negative.

Discussion

While repeated testing indicated the presence of *Las* in seedlings 3 to 9 months after emergence grown from seed, no plants ever developed HLB symptoms. Thus, the earlier report (Tirtawidjaja, 1981) of HLB symptoms in seedlings from seeds harvested from HLB-affected trees was not confirmed. Our negative findings for HLB symptom development and repeatable detection of *Las* by qPCR for 3,153 seedlings of various scion and rootstock cultivars are supported by the recent report by Hartung et al. (2010) of a survey of 723 citrus seedlings from HLB-affected fruit that failed to detect transmission of *Las* in seedlings after 3 years. Albrecht and Bowman (2009) also reported negative results for 686 rootstock and 431 'Valencia' orange seedlings tested by standard PCR over a period of 4 to 7 months. As in our study, Albrecht and Bowman (2009) germinated seedlings from seeds collected from *Las*-infected rootstock seed source trees in Florida. In both of these previous reports, symptoms of HLB were not observed in any of the seedlings. As in our study, Albrecht and Bowman (2009) reported weakly positive PCR results from two rootstock seedlings and three 'Valencia' seedlings that could not be confirmed by subsequent assays. Tatineni et al. (2008) detected *Las* in seedcoats of seeds from infected citrus fruit, but the bacterium was not found in a small sampling of endosperm and embryos. Since DNA-based detection systems do not determine whether bacterial cells are living or dead, transient detection of *Las* in seedlings does not confirm that the bacterium has infected the plant. In the most recent report of *Las* in citrus fruit peduncles, seedcoats, and seedlings, Hilf (2011) could find no consistent association between detection of pathogen DNA by PCR in fruit and seedcoat tissues and detection in seedlings derived from the infected fruit. Once again, this study provided no conclusive evidence for seed transmission.

Another explanation for the transient detection in our study is that *Las* DNA contamination occurred in qPCR assays. This is unlikely, since in a few cases repeated positive detections were obtained from separate samples of leaf tissues from the same plant. It is more probable that DNA contamination of the germinating seedling from the seedcoat was the source of qPCR detection. The likely cause of transient detection is the expected dilution and degradation of this DNA as the seedling developed.

In conclusion, the preponderance of findings for lack of transmission of *Las* and development of HLB symptoms in seedlings derived from seeds from the parent tree should be taken into account by citrus nurserymen and regulatory officials in formulating plans for citrus nursery tree production and plant protection guidelines.

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